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During transformation, ribosome synthesis increases concurrently with the size and number of nucleoli. U3 snoRNA-associated complexes are involved in the cleavage of pre-rRNA and 40S preribosome assembly and contain a number of proteins whose roles in the complex are unclear. Our research focused on characterizing the functional roles of human U3 snoRNA-associated factors by examining their localization and dynamics in live cells. We found that these factors can be divided into subclasses based on their localization and dynamics and showed for the first time that U3 snoRNA-associated proteins shuttle between nucleoli and the nucleoplasm and between nuclei and the cytoplasm.

These studies have led us to propose a model for the assembly and function of U3 snoRNA-associated complexes. We also carried out more extensive studies of the U3 snoRNA-associated protein Sof1 which defined the protein domains necessary and sufficient for its localization, characterized its interactions, and disrupted its function. These studies have indicated that Sof1 has characteristics that distinguish it from other U3 snoRNA-associated proteins. Overall, these experiments have clarified the roles of Sof1 and other U3 snoRNA-associated factors in ribosome synthesis and have contributed to our understanding of ribosome synthesis as a whole

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INTRODUCTION

Breast cancer is a widespread disease with a high morbidity rate. The molecular and cellular changes that act in the development and progression of breast and other cancers are not yet well understood, although significant progress has made in the past a few decades. Since the nuclei of cells act as the cellular centers for gene expression regulation at both the transcriptional and post-transcriptional levels, it is no surprise that many cellular changes during the transformation process have been shown to occur at the molecular or supra-molecular levels of the nucleus. As the functions of these cells change, so do the molecular and structural makeup of their nuclei to facilitate these changes. Therefore, the dissection of the numerous nuclear changes that occur during the transformation process will undoubtedly contribute valuable knowledge towards our understanding of the disease.

One of the most easily visible nuclear changes that take place during transformation is an increase in the size and shape of nucleoli, a long-utilized diagnostic marker [1-3] that is the site of many steps of ribosome synthesis. Microscopy studies of the nucleolus have identified three distinct nucleolar substructures: fibrillar centers (FC), dense fibrillar components (DFC), and granular components (GC) [4,5]. Ribosome synthesis begins at the FCs and subsequent steps take place in DFCs and then the GC [6]. This process is closely controlled and requires a large number of trans-acting factors that act to transcribe, process, and assemble preribosomal particles in the nucleolus [7,8]. U3 small nucleolar RNA (snoRNA) and associated proteins are required for the processing of preribosomal RNA (pre-rRNA) and assembly of preribosomes. There are two major U3 snoRNA containing complexes. The U3 monoparticle contains the core Box C/D snoRNA-associated proteins, which include fibrillarin, Nop56, Nop58, and U3-55K [9]. An early preribosome associated complex which carries out the processing and assembly functions contains the monoparticle and additional factors that we refer to as preribosome associated proteins, a group that includes Imp3, Imp4, Mpp10, Rcl1, and Sof1 [10,11]. The means by which these complexes are assembled, how they function, and the roles of the majority of the components in these complexes are not well understood. To address these questions, we have examined the dynamics and distribution of human U3 complex associated components in cells and begun to characterize the interactions undertaken by these components.

BODY

RESEARCH PROGRESS

The revised statement of work submitted last year, which focused on the cloning and characterization of the human preribosome associated protein hSof1, has been followed and has led into a broader investigation of other components of U3 snoRNA-containing complexes. My research on these components has been divided into five main goals: (1) Cloning of U3 snoRNA complex proteins and production of anti-hSof1 antibodies; (2) Localization and dynamics studies of U3 snoRNA complex proteins; (3) Characterization of hSof1 interactions; (4) Characterization of hSof1 function(s); (5) Determination of hSof1 tertiary structure.

Cloning of U3 snoRNA-associated complex proteins and antibody preparations.

Cloning of the human homologs of many of the U3 snoRNA-associated complex proteins and production of anti-hSof1 antibodies was necessary for the characterization of these proteins. The cDNA of the human homologs of Imp3, Imp4, Rcl1, and Sof1 was cloned from HeLa (human cervical cancer) cells and other clones were generously provided by co-workers or other labs. Recombinant fragments of hSof1 were used to produce and purify polyclonal antibodies and other antibodies were either purchased or generously provided by other researchers.

The subcellular localization of components of U3 complexes

Localization and dynamics studies of components of U3 complexes were undertaken to determine the localization of these complexes and to gain insight into the mode of their function and the functions of the individual components. *In situ* hybridization studies of U3 snoRNA and immunofluorescence studies of either endogenous proteins or of GFP- or FLAG-tagged proteins were used to assay subnuclear localization. These experiments found that U3 snoRNA and its core binding proteins, including fibrillarin, Nop56, Nop58, and U3-55K, are primarily concentrated in the FCs and DFCs of active nucleoli and exhibit a weaker label in the GC. In contrast to U3 snoRNA and core proteins, preribosome associated proteins GFP-Imp3, -Imp4, -Mpp10, -Rcl1, and –Sof1 were predominantly concentrated in GCs and the labels were lower within the FCs and DFCs. The differential localization of these two classes of factors was maintained when RNA polymerase I, and thus ribosome synthesis, was inhibited. Though the predominant localizations of these two sets of factors differ, there are significant areas of overlap that may represent the sites where they reside as a single complex. These results led us to propose a model in which U3 monoparticles associate with the fibrillar components (FCs and DFCs) of nucleoli and bind pre-rRNA during transcription, triggering recruitment of preribosome associated proteins to assemble the complex necessary for pre-rRNA processing.

Further characterization of hSof1 localization was done using deletion mutagenesis. Immunofluorescent analyses of tagged deletion constructs showed that the carboxy-terminus of hSof1 was necessary and sufficient for wild-type localization. Explorations into the interactions undertaken by these mutants are ongoing.

Intracellular dynamics of U3 snoRNA-associated proteins

In addition to the analyses of the steady-state intracellular localization of U3 snoRNA-associated proteins, we have also examined the dynamics of these proteins in living cells. Fluorescence recovery after photobleaching (FRAP) assays done on HeLa cells transfected with GFP-fibrillarin or hSof1 showed that the nucleolus localized populations of both proteins exchange rapidly with the pools in the nucleoplasm, though hSof1 moves at a much faster rate and does so independent of ribosome or protein synthesis. These studies showed that U3 snoRNA-associated proteins move rapidly through nuclei in manners that may reflect their function.

To investigate whether these U3 snoRNA-associated proteins may also move out of nuclei, we used heterokaryon assays to investigate whether these proteins shuttle between nuclei and the cytoplasm. These assays showed for the first time that GFP-fibrillarin, -U3-55K, -Imp3,

-Imp4, -Mpp10, -Rcl1, and –Sof1 all shuttled between nuclei and the cytoplasm, though they shuttled at different rates. The core proteins shuttled at the slowest rates while the preribosome associated proteins shuttled at higher rates, indicating that these groups of proteins may shuttle as members of different complexes. The shuttling of these proteins did not require either ongoing ribosome synthesis or ribosome export pathways. In addition, hSof1 shuttled at a significantly higher rate than any other protein tested, indicating that it may be shuttling as an individual protein or as part of different complexes that those formed by the other proteins. Though we do not know the function of the shuttling of these proteins, we speculate that these such shuttling could be part of a regulatory pathway or that these proteins could modulate ribosome export.

Functional interactions with other cellular components

We have been interested in identifying cellular factors that interact with hSof1 with hopes of gaining insight into its function. Since hSof1 is part of U3 snoRNA-containing preribosome and contains WD40-repeats, domains that can facilitate interactions with proteins and RNAs, it is likely that it makes one or more functional interactions. These interactions are currently being explored using immunoprecipitation and have thus far verified its association with a preribosomal particle. However, we have preliminary evidence that hSof1 is much more weakly associated with U3 snoRNA than are other preribosome associated proteins, which, combined with the relatively high FRAP and nucleocytoplasmic shuttling rates of hSof1, may indicate that this protein in involved in different complexes than other preribosome associated proteins.

Disruption of Sof1 function

The fourth focus was on the characterization of Sof1 function by disrupting its activity using various approaches. Overexpression of GFP-tagged deletion constructs of hSof1 did not provide any dominant-negative mutants. Experiments in which anti-hSof1 antibodies were injected into HeLa cell nuclei did not have significant effects and the use of small interfering RNAs directed against hSof1 did not knockdown hSof1 protein levels or affect cell viability. We were able to knockdown the protein levels of the *Xenopus laevis* homolog of Sof1 (xSof1) in developing embryos using antisense technologies and showed that xSof1 is essential for the development of these embryos in a manner consistent with its role in ribosome synthesis.

Determination of hSof1 Tertiary Structure

The fifth approach to characterizing hSof1 was the characterization of its tertiary structure. We had established a collaboration with the lab of Dr. Wayne Anderson to determine the tertiary structure of recombinant hSof1 but were unable to produce sufficient soluble protein for use in X-ray crystallography and ceased this pursuit.

Summary

Overall, these five primary goals have increased our understanding of the roles of U3 snoRNA-associated proteins in ribosome biogenesis and clarified the relationship between hSof1 and this process. These findings will have contributed to our understanding of ribosome synthesis and may provide insight into the relationship between ribosome synthesis and transformation.

TRAINING PROGRESS

Significant advances in training have been made. As described above, my studies have required the use of multiple techniques. These have included molecular biological (RT-PCR, expression cloning, deletion mutagenesis), biochemical (protein expression and purification, immunoprecipitation, gradient fractionation), and cell biological (localization studies, nucleocytoplasmic shuttling assays, FRAP, siRNA) approaches.

Through my graduate career, I stayed up to date on the current research in the ribosomal, nuclear, cancer, and other fields. Besides reading current literature, I participated in a cancer biology journal club and attended both departmental and other lecture series. These activities keep me well informed on a broad range of biological research. Further education has taken place at several scientific meetings. I attended and presented posters at Era of Hope meeting in September, 2002, the annual meeting of the American Society for Cell Biology in December, 2002, and a Ribosome Synthesis meeting in June, 2003. The talks and posters contributed to my understanding of the latest findings on the nucleus and allowed me to make contacts with leading researchers in the field.

I am first author on a paper that is under review at Molecular Biology of the Cell and have been published as second author on a book chapter. Within the next year, we expect to publish one or more papers on my thesis research. In July 2003 I finished and successfully defended my Ph.D. thesis and will officially graduate in December of this year. I am entering medical school at the University of Iowa beginning in August and have plans to continue research in fields that will utilize my basic research and clinical skills.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning of human U3 snoRNA-associated proteins
- Production of anti-hSof1 antibodies
- Characterizing the localization U3 snoRNA-associated factors
- Characterizing the subnuclear and subcellular dynamics of U3 snoRNA-associated factors
- Characterization of the protein and RNA interactions of hSof1
- Demonstrating that *Xenopus* Sof1 is essential for embryonic development

Reportable Outcomes

1. Poster presentation at the Era of Hope Meeting, September, 2002. Poster entitled "hSof1p, a U3 snoRNP component, is associated with cytoplasmic ribosomes."
2. Poster presentation at the Meeting at the American Society of Cell Biology, December, 2002. Poster entitled "hSof1p, Human Homolog of a Yeast U3 snoRNP Component, is Associated with Cytoplasmic Ribosomes"
3. Poster Presentation at the Ribosome Synthesis Meeting, June, 2003. Poster entitled "Components of the SSU Processome are Nucleocytoplasmic Shuttling Proteins"
4. Poster Presentation at the Ribosome Synthesis Meeting, June, 2003. Poster entitled "UBF is Essential in *Xenopus* Embryonic Development and May Function Outside of RNA Polymerase I-Mediated Transcription."
5. Book Chapter: R. Kamath, D. J. Leary, and S. Huang. Nuclear Components and Tumor Markers. *In* Visions of the Nucleus – Eukaryotic DNA. P. Hemmerich and S. Diekmann, editors. American Scientific Publishers. 2003.
6. Research Article: D. J. Leary, M. Terns, and S. Huang. Components of U3 snoRNA Containing Complexes Shuttle Between Nuclei and the Cytoplasm and Differentially Localize in Nucleoli: Implications for Assembly and Function. *Mol. Biol. Cell*, Under Review.

Conclusions

Significant progress has been made in characterizing the structure and function of hSof1 and the localization and dynamics of hSof1 and other U3 snoRNA-associated proteins. This research has provided me with extensive laboratory experience and has yielded several publications. In addition, my active involvement in journal clubs, scientific meetings, and writing have kept me well-informed of advances in my field. I believe that together these aspects of my doctoral training have prepared me well for future research and my forthcoming education in medical school.

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